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(54) Title: IMPROVED TREATMENT METHOD FOR CANCER

(57) Abstract

The *in vivo* chemotherapeutic treatment of cancer cells in a living animal is improved by first administering to the animal an antagonist specific for intracellular histamine in an amount sufficient to inhibit the binding of intracellular histamine in normal cells. An enhanced toxic effect on the cancer cells from the chemotherapeutic agent is obtained while any adverse effect of the chemotherapeutic agent on normal cells is inhibited. In addition, long term continuous administration of the antagonist following administration of the chemotherapeutic agent results in at least amelioration of the adverse side effects of chemotherapy. The treatment of cancer cells using DPPE specifically illustrates the invention.

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+ DESIGNATIONS OF "SU"

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IMPROVED TREATMENT METHOD FOR CANCER

FIELD OF INVENTION

The present invention relates to the improved treatment of cancer in animals, including humans, using chemotherapeutic agents.

BACKGROUND TO THE INVENTION

One of the major chemotherapeutic treatments is that of malignant growth (cancer) in humans. The objective of chemotherapy is the total extermination of clonogenic tumor or malignant cells, with minimal damage to the patient. However, one of the major limitations of the chemotherapeutic approach for managing human cancer is the general inability of anticancer drugs to discriminate between normal and tumorous cells. Anti-neoplastic agents have the lowest therapeutic indicies of any class of drugs used in humans and hence produce significant and potentially life-threatening toxicities. Certain commonly-used anti-neoplastic agents have unique and acute toxicities for specific tissues. For example, the vinca alkaloids possess significant toxicity for nervous tissues, while adriamycin has specific toxicity for heart tissue and bleomycin has for lung tissue. In general, almost all members of the major categories of anti-neoplastic agents have considerable toxicities for normal cells of gastrointestinal, epidermal and myelopoietic tissues.

Generally, the dose-limiting consideration for chemical management of cancer in humans is the toxicity that anti-neoplastic agents have for the pluripotent stem cells of myelopoietic tissue. This toxicity arises from the fact that most anticancer drugs function preferentially against proliferating cells but with no significant capacity to discriminate between cycling normal and cycling tumor tissues.

Attempts have been made to confer specificity upon presently-available chemotherapeutic agents. In Anticancer Research 6:451 to 464 (1986), Robert C. Warrington describes certain in vitro and in vivo experiments demonstrating the achievement of improvements in both the specificity and efficacy of a number of commonly-used anticancer drugs by using these agents in combination with L-histidinol. L-histidinol is a structural analogue of the essential amino acid, histidine, in which the α-carboxyl group has been reduced to a primary alcohol. In the work presented by Warrington in this paper, L-histidinol was found to be effective at doses of approximately 1000 mg/kg of tissue administered five hours or more prior to the chemotherapeutic agent.

SUMMARY OF INVENTION

It now surprisingly has been found that, if an antagonist specific for a recently-discovered intracellular histamine receptor, designated H_{IC}, different from traditional histamine receptors classified as H_1, H_2 or H_3, is administered to a living animal having cancer, then the specificity and efficacy of chemotherapeutic agents for cancer cells is improved. By employing an antagonist specific to inhibit the binding of intracellular histamine, the improved effect is obtained at significantly lower dosage levels administered at a significantly shorter period of time prior to administration of the chemotherapeutic agent than is shown in the Warrington work referred to above.

The present invention is broadly applicable to the treatment of malignant cells in a living animal where the administration of chemotherapeutic agents normally adversely affects the normal cells in the animal. By first administering to the animal an antagonist specific for intracellular histamine in an amount sufficient to inhibit binding of intracellular histamine in normal

cells at the intracellular histamine binding site, the specificity and efficacy of subsequently administered therapeutic agents on malignant cell is improved.

Accordingly, in one aspect of the present invention, there is provided a method for the treatment of cancer cells in a living animal, which comprises (a) administering to the animal an antagonist specific for intracellular histamine in an amount sufficient to inhibit the binding of intracellular histamine in normal cells, and (b) subsequently administering to the animal at least one chemotherapeutic agent in an amount toxic for the cancer cells, whereby an enhanced toxic effect on the cancer cells from the at least one chemotherapeutic agent is obtained while any adverse effect of the at least one chemotherapeutic agent on the normal cells is inhibited.

The present invention also includes a kit for the treatment of cancer cells in a living animal and useful in the above-described aspect of the invention. The kit comprises (a) a first component consisting of an antagonist specific for intracellular histamine in a dosage amount sufficient to inhibit the binding of intracellular histamine in normal cells of the animal, and, separately; (b) a second component consisting of at least one chemotherapeutic agent for the cancer cells in a dosage amount toxic to the cancer cells.

The invention further comprises the use of an antagonist specific for intracellular histamine to obtain an enhanced toxic effect of a chemotherapeutic agent on cancer cells in a living animal while inhibiting any adverse effect of the chemotherapeutic agent on normal cells in the animal.

It has further been found that, if a patient being treated with a chemotherapeutic agent also is given an IV infusion over a period of from about 24 to about 72 hours after administration of the chemotherapeutic

agent, then the side effects generally associated with chemotherapy, namely nausea, vomiting, anorexia and stomatitis, are at least ameliorated and often prevented.

Accordingly, in another aspect of the present invention, there is provided a method for the treatment of cancer cells, which comprises (a) administering to the animal at least one chemotherapeutic agent in an amount toxic for the cancer cells, and (b) administering to the animal for a period of at least twenty-four hours an antagonist specific for intracellular histamine in an amount sufficient to inhibit the binding of intracellular histamine in normal cells, whereby the side effects of administration of the chemotherapeutic agent are at least ameliorated.

The invention further includes a kit useful in the latter procedure, comprising the kit referred to above and an antagonist specific for intracellular histamine in a dosage amount sufficient to ameliorate the side effects of administration of the chemotherapeutic agent to the animal.

The invention additionally comprises The use of an antagonist specific for intracellular histamine to ameliorate the side effects of administration of a chemotherapeutic agent for cancer cells to a living animal.

BRIEF DESCRIPTION OF DRAWINGS

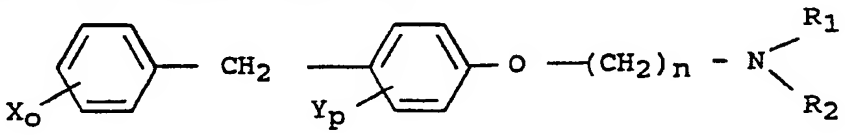
Figures 1 to 5 are graphical representations of test data generated in certain experiments set forth in the Examples below.

GENERAL DESCRIPTION OF INVENTION

In the present invention, any compound which is an antagonist specific for the intracellular histamine receptor is useful and is administered in an amount sufficient to inhibit the binding of intracellular

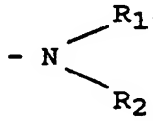
histamine at the intracellular binding site (H_{IC}) in normal cells.

Specific compounds which are useful in the present invention are diphenylmethanes of the formula:

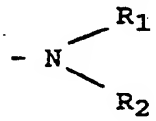


wherein X and Y are each chlorine or bromine, o and p are 0 or 1, R_1 and R_2 are each alkyl groups containing 1 to 3 carbon atoms or are joined together to form a hetero-ring with the nitrogen atom and n is 1, 2 or 3. Pharmaceutically-acceptable salts of the diphenylmethanes may be employed.

In one preferred embodiment, the group



is a diethylamino group and, in another preferred embodiment, a morpholino group. o and p are usually 0 and n may be 2. In one particularly preferred embodiment, n is 2, o and p are each 0 and



is a diethylamino group. This compound, namely N,N-diethyl-2-[4-(phenylmethyl)-phenoxy]ethanamine, in the form of its hydrochloride salt, is abbreviated herein as DPPE.

The compounds used herein are antagonists specific for intracellular histamine and specifically inhibit intracellular histamine binding at a site designated H_{IC}. L-histidinol used in the Warrington work referred to above, is not <u>specific</u> for intracellular histamine. Although some affinity for binding to H_{IC} is exhibited by L-histidinol, this compound also binds to other

histamine binding sites. The present invention employs compounds which bind only to H_{IC}.

In the present invention, significantly-smaller quantities of the antagonist compound are used when compared to L-histidinol (typically 2 mg/kg vs. 1000 mg/kg) and the antagonist compound is administered in the present invention a much shorter period before the chemotherapeutic agent(s) when compared to L-histidinol (typically 20 to 30 minutes vs. 5 hours).

The antagonist compound employed in the present invention is administered to the patient in any convenient manner, such as by injection of a solution thereof in an aqueous pharmaceutically-acceptable vehicle.

The antagonist compound is administered to the patient before administration of at least one chemotherapeutic agent. The chemotherapeutic agent or more commonly a mixture of such agents may be administered in any convenient manner consistent with its normal manner of administration following conventional chemotherapeutic practice.

The administration of the antagonist compound to the patient prior to administration of the chemotherapeutic agent is necessary in order to permit the antagonist to inhibit the binding of intracellular histamine in normal cells and thereby, in effect, shut down proliferation of the normal cells.

The length of time prior to administration of the chemotherapeutic agent that the antagonist compound is administered depends on the antagonist compound, its mode of administration and the size of the patient. Generally, the antagonist compound is administered to the patient about 15 to about 90 minutes, preferably about 30 to about 60 minutes, prior to administration of the at least one therapeutic agent.

The quantity of antagonist compound administered to the patient should be at least sufficient to inhibit binding of intracellular histamine in normal cells. The quantity required to achieve the beneficial effects of the present invention depends upon the antagonist compound employed, the chemotherapeutic agent employed and the quantity of such agent employed.

In general, the quantity of antagonist compound employed is from about 2 to about 75 mg/kg of animal to which the antagonist compound is administered. The present invention is able to achieve an enhanced chemotherapeutic effect on cancer cells while, at the same time, also protecting normal cells from damage by the chemotherapeutic agent in a wide variety of circumstances where traditional chemotherapy leads to damage of normal cells or tissues not involved in the disease process. Examples of the adverse effects on normal cells which result in traditional chemotherapy include:

(a) the killing of, or damage to, bone marrow cells,

(b) the killing of, or damage to, normal cells lining the gastrointestinal tract,

(c) the killing of, or damage to, normal heart muscle cells by anti-cancer drugs, such as doxorubicin (Adriamycin) and its analogues, including daunorubicin and epirubicin, and the non-related but cross-reactive compound, mitoxantrone, and

(d) damage to normal tissue, particularly in the kidneys by cisplatinum.

In cancer-bearing animals, DPPE treatment alone demonstrates little, if any, <u>in-vivo</u> effect on tumor growth. However, when combined with known anti-cancer drugs, a marked synergistic action is observed whereby tumors are inhibited or killed. This effect has led,

for example, to marked regressions or cures in some animal cancers, such as sarcoma and melanoma.

As noted above, continued administration of the antagonist compound following administration of the chemotherapeutic agent, specifically up to about 0.2 mg/kg of DPPE on a daily basis, at least ameliorates, and often eliminates, the side effects often associated with chemotherapy, including nausea, vomiting, anorexia and stomatitis, and preferably is effected herein, with the longer the period of administration, the more significant is the protection against the side effects. A daily dose of about 0.1 to about 5 mg/kg of such antagonist may be employed.

Such continued administration of antagonist component is most conveniently effected by intravenous administration, although oral administration may be feasible and, in some cases, more desirable from the standpoint of patient acceptance and of decreasing the load on the medical facility.

THEORY

While the applicant does not wish to be bound by any theory to explain the beneficial effects achieved by the present invention, the following theory is proposed. The compound administered to the patient is a specific antagonist of histamine binding at a newly-discovered novel intracellular receptor (H_{IC}) (see, for example, "Histamine is an Intracellular Messenger Mediating Platelet Aggregation" by Saxena et al, Science, Vol 243, pg. 1596-1599). Intracellular histamine normally functions through this receptor to mediate or modulate many important cell functions, including cell proliferation, immune responses and platelet aggregation.

Protection of the normal cells is achieved through antagonism of histamine at H_{IC} by the antagonist. Such antagonism results in a temporary complete shut-down of

cell division, so that normal cells are not susceptible to DNA damage in the presence of the chemotherapeutic agent(s), which preferentially attack dividing cells. In this way, for example, DPPE is effective to block therapy-associated toxicity of normal bone marrow stem cells.

In addition, the antagonism results in an increase in the levels of prostaglandins (natural substances which are known to protect tissues from various injurious agents) in the tissue. For example, DPPE treatment results in an increase by 500% in prostaglandin (PG)I_2, a protective prostaglandin, in the gut. Through this mechanism, DPPE is known to completely block ulcer formation in the presence of noxious agents, such as alcohol and cysteamine (see U.S. Patent No. 4,829,068 in which I am a co-inventor).

The antagonist further effects a potent blockage of the degranulation of tissue mast cells, whose granular contents, including histamine itself, have been linked to tissue damage and severe systemic side effects. Certain anti-cancer drugs, such as adriamycin, cause significant mast cell degranulation, an effect which has been related to cardiotoxic effects.

As with bone marrow cells, the treatment of normal proliferating lymphocytes (immune cells), according to the invention, results in a dose-dependent blocking of DNA synthesis and a shut-down of these cells without causing cytotoxicity. The antagonist has an effect on both T-lymphocytes and B-lymphocytes in the immune system. For example, DPPE is able to completely antagonize proliferation of T-lymphocytes in the presence of Concanavalin A, a potent mitogen and plant lectin. DPPE also blocks the stimulation of antibody formation by the mediator interleukin-2 in certain B cells, resulting is a decrease in antibody formation.

In contrast to its cytoprotective effect on normal cells and tissue in vivo, as described herein, DPPE treatment damages and/or kills malignant cells in vitro, as described in U.S. Patent No. 4,803,227, or those which are virally infected.

EXAMPLES

Example I

This Example illustrates in vivo augmentation by DPPE of adriamycin anti-tumor activity in a murine sarcoma model.

C-3 fibrosarcoma cells (3×10^5) were injected into the left gluteal region of C3H mice on day 0. On day 1, the mice were provided with treatment by a combination of DPPE and adriamycin, administered intraperitoneally. The DPPE was administered 60 minutes prior to administration of the adriamycin. Mice also were administered with saline, DPPE alone and adriamycin alone.

Animals in the experiments (n=12) were followed for 60 days. At the end of the experimental period, those animals free of palpable tumors were considered cured.

The results obtained are set forth in the following Table I:

TABLE I

Treatment	Number of Rats Tumor-Free (n=12)
Saline	1
Adriamycin (2mg/kg)	0
DPPE (50mg/kg)	0
DPPE (2mg/kg) / Adriamycin (2mg/kg)	1
DPPE (25mg/kg) / Adriamycin (2mg/kg)	3
DPPE (50mg/kg) / Adriamycin (2mg/kg)	7

It will be seen from the results set forth in the above Table I, that, when adriamycin and DPPE alone are administered, no effect was obtained whereas when

increasing quantities of DPPE were employed in combination with adriamycin, an increased anti-tumor activity was observed, such that, at the highest dose of DPPE tested (50mg/kg), 7 out of the 12 animals were cured.

Example II

This Example illustrates protection of bone marrow progenitors by DPPE in mice treated with a lethal dose of 5FU and adriamycin.

Mice of the strain C57B1 were administered a lethal dose (7.5mg) of 5FU (5-fluorouracil), DPPE (100mg/kg) or a combination of a lethal dose of 5FU and DPPE (100mg/kg or 4mg/kg) and the results were compared with a control group to which saline only was administered. The DPPE and saline were administered 90 minutes prior to the 5FU. Bone marrow cell counts were made at 24 hours and 48 hours post administration.

The results obtained are set forth in the following Table IIA:

TABLE IIA

Treatment	CFU-C/10^4 cells (1)		R.C.S.(2)	
	24h	48h	24h	48h
Saline	38.3	40.3	1.0	1.0
5FU	0.2	0.09	0.006	0.002
DPPE	36.7	38.6	0.96	0.96
DPPE (100mg/kg) + 5FU	35.7	37.3	0.93	0.93
DPPE (4mg/kg) + 5FU	38.3	33.3	1.0	0.83

Note:

(1) No. of bone marrow colonies. CFU-C = colony-forming units in culture

(2) Relative Cell Survival

Experiments paralleling those described above with 5FU were carried out employing a lethal dose of adriamycin (20mg/kg). The results obtained from these experiments are set forth in the following Table IIB:

TABLE IIB

Treatment	CFU-C/10^4 cells		R.C.S.	
	24h	48h	24h	48h
Saline	43.7	42.7	1.0	1.0
Adriamycin	1.8	0.65	0.04	0.006
DPPE (4mg/kg)	42.3	41.3	0.97	0.97
DPPE (4mg/kg) / Adriamycin	39.7	39.0	0.91	0.91
DPPE (100mg/kg) / Adriamycin	43.3	41.3	0.99	0.97

As may be seen from the results set forth in the above Tables IIA and IIB, the administration of the DPPE along with the 5FU or adriamycin provided almost complete protection for bone marrow progenitors from the lethal effects of the 5FU or adriamycin.

EXAMPLE III

This Example illustrates in vivo augmentation of BCNU anti-tumor activity in a B16 melanoma lung metastasis model.

5 x 10^4 B16 melanoma cells were injected intravenously into the tail vein of C57Bl mice at day 0. The mice were treated with either saline, 32mg/kg of DPPE, 1mg of BCNU or a combination of 32mg/kg of DPPE and 1mg of BCNU, by intraperitoneal injection on day 1. The DPPE was administered 60 minutes before the BCNU.

In each group, six of the twelve animals were sacrificed at day 14 and lungs were removed for determination of metastasis. The remaining six animals were followed to death. The numbers and size of the lung metastases were determined by visual or microscope count.

The results obtained are set forth in the following Table III:

TABLE III

Treatment	Numbers and (% control) of lung metastases		Size of lung metastases (1)	Median Survival (days)
Saline	241	-	All Macro	19
DPPE	219	(91%)	All Macro	21
BCNU	144	(60%)	All Macro	24
DPPE/BCNU	58	(27%)	All Micro	32

(1) Macro means visually determined. Micro means microscopically only.

As may be seen from the results in Table III, the inhibitory effect of BCNU on the lung tumors was significantly increased by the additional presence of DPPE, which itself alone had a marginal effect.

EXAMPLE IV:

This Example illustrates in vivo augmentation of daunorubicin anti-tumor activity in a B16 melanoma lung metastasis model.

The procedure of Example III was repeated employing daunorubicin in place of BCNV. Groups of six mice were injected with B16f10 melanoma cells and 24 hours later received saline, 4 mg/kg of DPPE alone, a non-lethal dose of daunorubicin alone (12.5 mg/kg) or DPPE (4, 25 or 50 mg/kg) one hour prior to daunorubicin (12.5 mg/kg). All animals were followed to death or for 60-days post injection, and sacrificed for lung metastases.

The results obtained are set forth in the following Table IV:

TABLE IV

Treatment Group	Median Survival (days)	No. Cures (n=6)
Saline	17	0
Daunorubicin (12.5 mg/kg)	25	0
DPPE (4 mg/kg) + Daunorubicin (12.5 mg/kg)	29	2
DPPE (25 mg/kg) + Daunorubicin (12.5 mg/kg)	60^{+}	4
DPPE (50 mg/kg) + Daunorubicin (12.5 mg/kg)	60^{+}	4

As may be seen from the results of Table IV, the inhibitory effect of daunorubicin or lung tumors was enhanced by the presence of DPPE.

EXAMPLE V:

This Example shows in vivo host cytoprotection from a lethal dose of adriamycin.

Saline or DPPE (2 mg/kg) were administered to DBA/2 mice 1 hour (n=12) or 15 minutes (n=6) prior to administration of 15 mg/kg of adriamycin. The number of survivors after 30 days was determined. The results are set forth in the following Table V:

TABLE V

Treatment	Number of Survivors
Saline	4/12 (33%)
DPPE	13/18 (72%)

As may be seen from Table V, the administration of DPPE provided in vivo host cytoprotection from the lethal dose of adriamycin.

EXAMPLE VI:

This Example illustrates the effect of DPPE on thymidine incorporation into lymphocyte DNA.

Spleen cells from BALB/C mice were stimulated with Concanavalin A (5 μg/ml) for thymidine incorporation. The cells then were treated with varying does of DPPE and the level of thymidine incorporated was determined. The results were plotted graphically and appear as Figure 1. As may be seen from this Figure, at a dosage level of 25 μM, DPPE completely blocks thymidine incorporation into DNA but does not adversely affect cell survival. Thus, the DPPE treatment puts normal proliferating lymphocytes into a state of growth arrest without causing cytotoxicity.

The experiment was repeated using 2.5 μg/ml of Concanavalin A in place of 5 μg/ml and 2% fetal calf serum in place of 10%. The results obtained are illustrated in Figure 2. At concentrations of DPPE which inhibited DNA synthesis (5 μm), no significant cytotoxicity was observed.

EXAMPLE VII:

This Example also illustrates the effect of DPPE on thymidine incorporation into lymphocyte DNA.

The experiment of Example VI was repeated employing virally-infected non-senescing transformed spleen-derived lymphocytes (S-10) also of BALB/C origin with 0.25 nM of ^{3}H-thymidine added. The results were plotted graphically and appear as Figures 3 and 4 respectively. As may be see therein, in contrast to Figures 1 and 2, 25 μM of DPPE caused approximately 50% cytotoxicity to the virally-infected cells. When adjusted for cell number, thymidine incorporation increased at cytotoxic concentrations of DPPE (10 to 25 μM).

The experiment was again repeated using human breast cancer cell (MCF-7) with 0.25 nM of thymidine added. The results were plotted graphically and appear

as Figure 5. Analogous results can be seen to those observed with the S-10 cells.

Example VIII:

A clinical study was carried out with 14 patients with advanced cancer.

(a) DPPE alone was administered to patients to determine a safe dose range in humans. The highest non-toxic dosage was found to be 4 mg/kg given intravenously over a one-hour period. At 6 mg/kg over one hour, CNS toxicity (as manifested by any or all of muscle twitching, a drop of 1 to 2°C in body core temperature, auditory hyperacusis or hallucination, choreoathetosis, cerebellar ataxia and projectile vomiting) was dose limiting. However, significant toxicity was absent when 6 mg/kg was administered IV over 2 hours, suggesting that peak serum level determines CNS toxicity. When the dose is converted to mg/M^2, threshold for CNS toxicity occurs at the same dose previously observed in preclinical toxicology (ip route) studies in mice (240 mg/M^2).

(b) DPPE at a daily dose of 0.2 mg/kg, given as an IV infusion over 24 to 72 hours was found to be entirely without clinical side effects, with the possible exception of constipation in occasional patients, and not to cause any significant changes in biochemistries or blood counts. This dose of DPPE also has been determined to potently prevent, or ameliorate by over 90%, nausea, vomiting, anorexia and stomatitis in 6/6 patients treated with Adriamycin (60 mg/M^2). GI protection was most pronounced when DPPE was given at a dose of 0.2 mg/kg daily for 72 hours by IV infusion.

(c) Higher single IV doses of DPPE (1, 2, 4 mg/kg) given over 1 hour also appear to be significantly anti-emetic against Adriamycin, although some patients experienced nausea, or transient anorexia, at 4 mg/kg of DPPE alone. At doses of 1 to 6 mg/kg IV over 1 hour,

DPPE alone also was found to cause a transient decrease (20 to 30%) in neutrophil counts in 4/6 patients, with complete recovery by day 5 to 7. No significant effect of DPPE alone on platelets, hemoglobin or biochemistry has been observed.

(d) Using 0.2 mg/kg of DPPE as a daily dose, increased duration of treatment improved the therapeutic benefit to prevent nausea, vomiting, anorexia and a drop in nadir white counts, but not alopecia, caused by Adriamycin at a dosage level of 60 mg/M^2. A 24-hour DPPE infusion was an effective anti-emetic therapy in the first 24 to 48 hours following Adriamycin administration, but many patients then experienced delayed nausea, vomiting and/or anorexia at 72 or 96 hours after Adriamycin administration. However, when given as a 72-hour infusion, DPPE was observed to block completely all acute and delayed gastrointestinal side effects of Adriamycin in four patients, each of which had received two such treatments. In addition, two additional patients experienced only one minor episode of nausea and/or vomiting in the first 24 hours following Adriamycin administration, but then were well without any need for antiemetics. The 72-hour infusion of low dose DPPE also prevented mouth ulceration in one patient who had previously experienced this symptom during all previous non-Adriamycin chemotherapy and DPPE/Adriamycin given in shorter schedules.

(e) As compared to other regimens of DPPE/Adriamycin, nadir polymorphonuclear WBC counts at 14 days appear to be highest in patients who received 0.2 mg/kg DPPE for 72 hours (1,285 ± 385; mean ± S.E.M.). Platelet counts have been uniformly above 150,000/mm^3 at Day 14.

(f) In fifteen evaluable patients, five major responses, two breast, one lymphoma and one medullary carcinoma of thyroid, have been documented.

SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides a novel approach to chemotherapeutic treatment of cancer whereby an enhanced in vivo effect of the chemotherapeutic agent is obtained while achieving protection of normal cells from the toxic effects of the chemotherapeutic agent. Modifications are possible within the scope of this invention.

CLAIMS

What I claim is:

1. A method for the treatment of cancer cells in a living animal, which comprises:

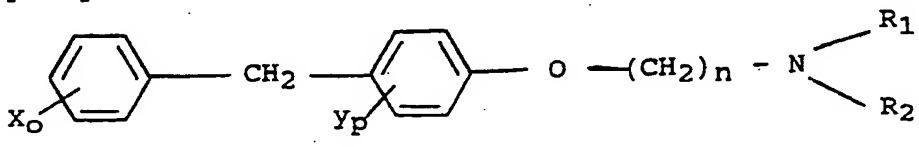
(a) administering to said animal an antagonist specific for intracellular histamine in an amount sufficient to inhibit the binding of intracellular histamine in normal cells, and

(b) subsequently administering to said animal at least one chemotherapeutic agent for the cancer cells in an amount toxic to said cancer cells,

whereby an enhanced toxic effect on said cancer cells from said at least one chemotherapeutic agent is obtained while any adverse effect of said at least one chemotherapeutic agent on said normal cells is inhibited.

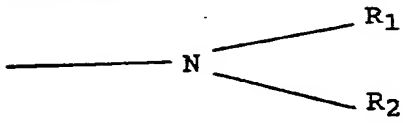
2. The method of claim 1 wherein, subsequent to said administration of said at least one chemotherapeutic agent, an effective amount of said antagonist is administered for a sufficient period of time to at least ameliorate the side effects of said administration of said at least one chemotherapeutic agent.

3. The method of claim 1 wherein said antagonist specific for intracellular histamine is a diphenylmethane of the formula:



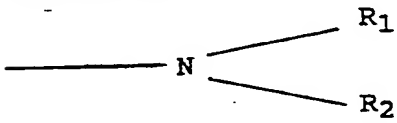
wherein X and Y are each chlorine or bromine, o and p are 0 or 1, R_1 and R_2 are each alkyl groups containing 1 to 3 carbon atoms or are joined together to form a hetero-ring with the nitrogen atom and n is 1, 2 or 3, or a pharmaceutically-acceptable salt thereof.

4. The method of claim 3 wherein the group



is a diethylamino group or a morpholino group.

5. The method of claim 3 wherein the group



is a diethylamino group, n is 2, and o and p are each 0.

6. The method of claim 5 wherein the diphenylmethane is in the form of a hydrochloride salt.

7. The method of claim 1 wherein said antagonist is administered to the animal about 15 to about 90 minutes prior to said administration of said at least one chemotherapeutic agent.

8. The method of claim 7 wherein the time is from about 30 to about 60 minutes.

9. The method of claim 7 wherein said antagonist is administered in an amount of from about 2 to about 75 mg/kg of animal.

10. A method for the treatment of cancer cells in a living animal, which comprises:

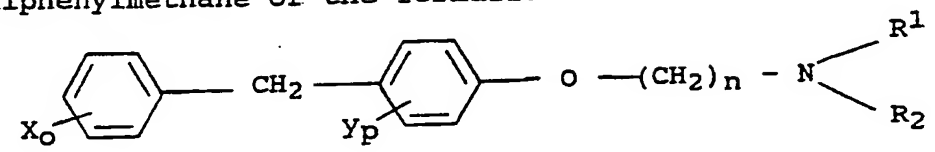
(a) administering to said animal at least one chemotherapeutic agent for the cancer cells in an amount toxic to said cancer cells, and

(b) subsequently administering over a period of at least twenty-four hours an antagonist specific for intracellular histamine in an amount sufficient to inhibit binding of intracellular histamine in normal cells,

whereby the side effects of the administration of the at least one chemotherapeutic agent are at least ameliorated.

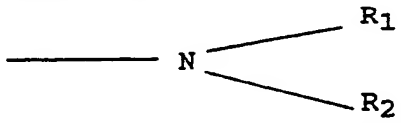
11. The method of claim 10 wherein said antagonist

specific for intracellular histamine is a diphenylmethane of the formula:



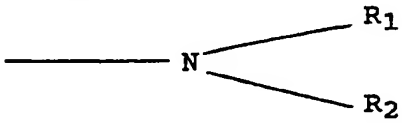
wherein X and Y are each chlorine or bromine, o and p are 0 or 1, R_1 and R_2 are each alkyl groups containing 1 to 3 carbon atoms or are joined together to form a hetero-ring with the nitrogen atom and n is 1, 2 or 3, or a pharmaceutically-acceptable salt thereof.

12. The method of claim 11 wherein the group



is a diethylamino group or a morpholino group.

13. The method of claim 10 wherein the group



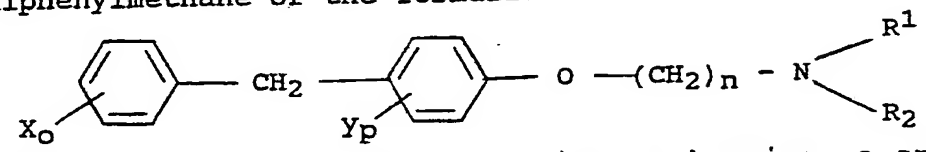
is a diethylamino group, n is 2, and o and p are each 0.

14. The method of claim 13 wherein the diphenylmethane is in the form of a hydrochloride salt.

15. The method of claim 14 wherein said antagonist is administered at a daily dosage of up to about 0.2 mg/kg and said administration thereof is effected for up to about 72 hours to achieve protection against nausea, vomiting, anorexia and stomatitis.

16. In a method of treatment of cancer cells in a living animal with at least one chemotherapeutic agent having an anti-cancer effect, the improvement which comprises:

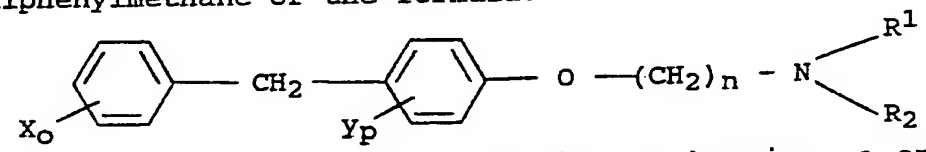
administering to said animal at least one diphenylmethane of the formula:



wherein X and Y are each chlorine or bromine, o and p are 0 or 1, R_1 and R_2 are each groups containing 1 to 3 carbon atoms or are joined together to form a hetero-ring with the nitrogen atom and n is 1, 2 or 3, in an amount of from about 2 to about 75 mg/kg of animal about 15 to about 90 minutes prior to administration of said at least one chemotherapeutic agent to said animal.

17. A method of protecting normal bone marrow cells in an animal from the adverse effects of at least one chemotherapeutic agent administered to the animal to treat cancer cells, which comprises:

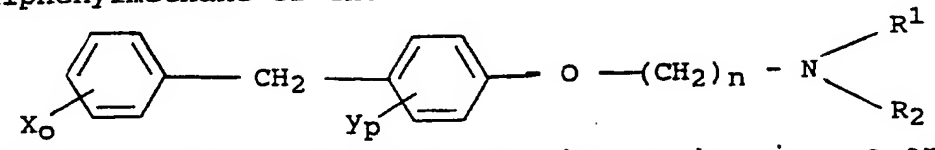
administering to said animal at least one diphenylmethane of the formula:



wherein X and Y are each chlorine or bromine, o and p are 0 or 1, R_1 and R_2 are each groups containing 1 to 3 carbon atoms or are joined together to form a hetero-ring with the nitrogen atom and n is 1, 2 or 3, in an amount of from about 2 to about 75 mg/kg of animal about 15 to about 90 minutes prior to administration of said at least one chemotherapeutic agent to said animal.

18. A method for protecting normal heart muscle cells in an animal from the adverse effects of at least one chemotherapeutic agent administered to the animal to treat cancer in the animal, which comprises:

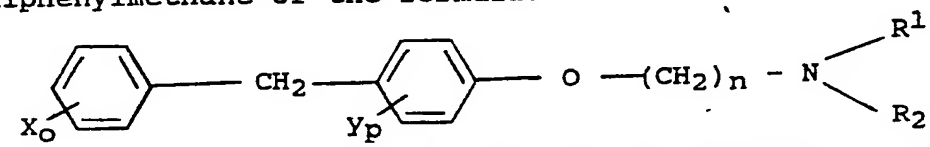
administering to said animal at least one diphenylmethane of the formula:



wherein X and Y are each chlorine or bromine, o and p are 0 or 1, R_1 and R_2 are each groups containing 1 to 3 carbon atoms or are joined together to form a hetero-ring with the nitrogen atom and n is 1, 2 or 3, in an amount of from about 2 to about 75 mg/kg of animal about 15 to about 90 minutes prior to administration of said at least one chemotherapeutic agent to said animal.

19. A method for protecting normal cells lining the gastrointestinal tract from the adverse effects of at least one chemotherapeutic agent administered to the animal to treat cancer cells, which comprises:

administering to said animal at least one diphenylmethane of the formula:



wherein X and Y are each chlorine or bromine, o and p are 0 or 1, R_1 and R_2 are each groups containing 1 to 3 carbon atoms or are joined together to form a hetero-ring with the nitrogen atom and n is 1, 2 or 3, in an amount of from about 2 to about 75 mg/kg of animal about 15 to about 90 minutes prior to administration of said at least one chemotherapeutic agent to said animal.

20. The method claimed in any one of claims 16, 17, 18 or 19 wherein said at least one diphenylmethane is DPPE.

21. A kit for the treatment of cancer cells in a living animal comprising:

(a) a first component consisting of an antagonist specific for intracellular histamine in a dosage amount sufficient to inhibit the binding of intracellular

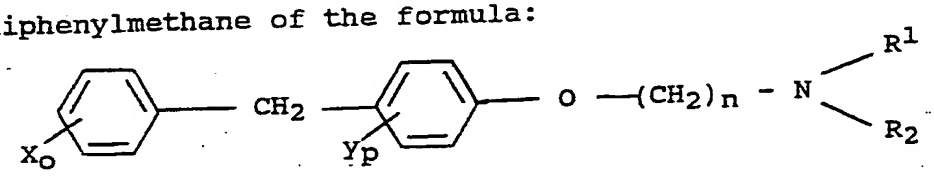
histamine in normal cells of the animal, and, separately,

(b) a second component consisting of at least one chemotherapeutic agent for the cancer cells in a dosage amount toxic to said cancer cells.

22. The kit of claim 21 further comprising, separately:

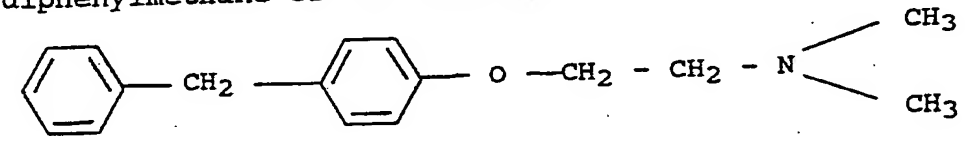
(c) a third component consisting of an antagonist specific for intracellular histamine in a dosage amount sufficient to ameliorate the side effects of administration of the chemotherapeutic agent to the animal.

23. The kit of claim 21 or 22 wherein said antagonist specific for intracellular histamine is a diphenylmethane of the formula:



wherein X and Y are each chlorine or bromine, o and p are 0 or 1, R_1 and R_2 are each alkyl groups containing 1 to 3 carbon atoms or are joined together to form a hetero-ring with the nitrogen atom and n is 1, 2 or 3, or a pharmaceutically-acceptable salt thereof.

24. The kit of claim 21 or 22 wherein said antagonist specific for intracellular histamine is a diphenylmethane of the formula:



optionally in the form of its hydrochloride salt.

25. The kit of claim 21 or 22 wherein said dosage amount of (a) is from about 2 to about 75 mg/kg of animal.

26. The kit of claim 21 or 22 wherein said dosage amount of (c) is up to about 0.2 mg/kg of animal.

27. The use of an antagonist specific for intracellular histamine to obtain an enhanced toxic effect of a chemotherapeutic agent on cancer cells in a living animal while inhibiting any adverse effect of the chemotherapeutic agent on normal cells in the animal.

28. The use of an antagonist specific for intracellular histamine to ameliorate the side effects of administration of a chemotherapeutic agent for cancer cells to a living animal.

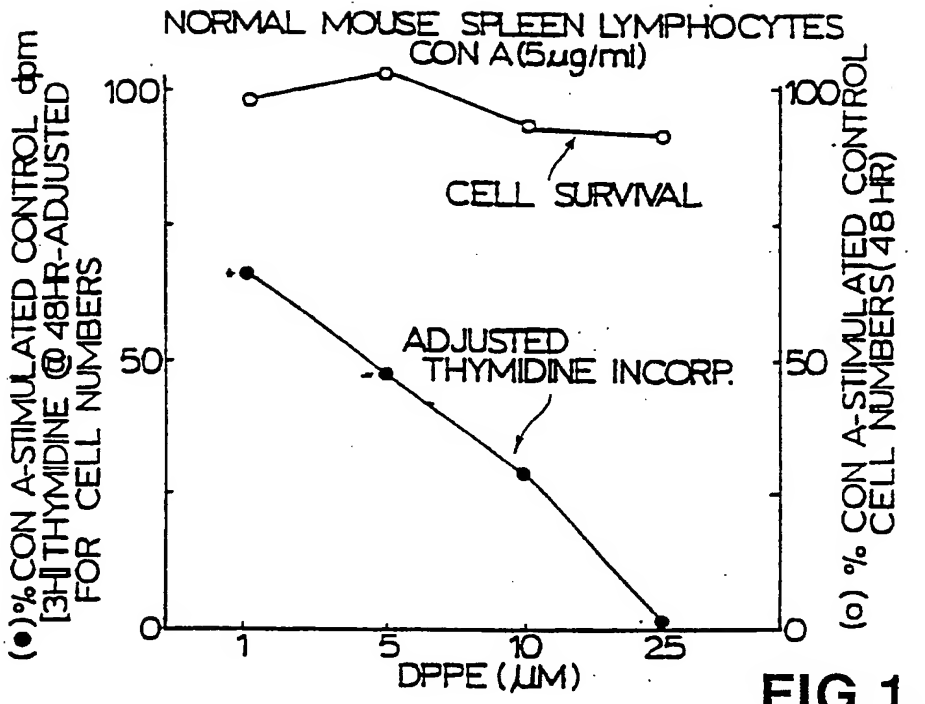


FIG.1.

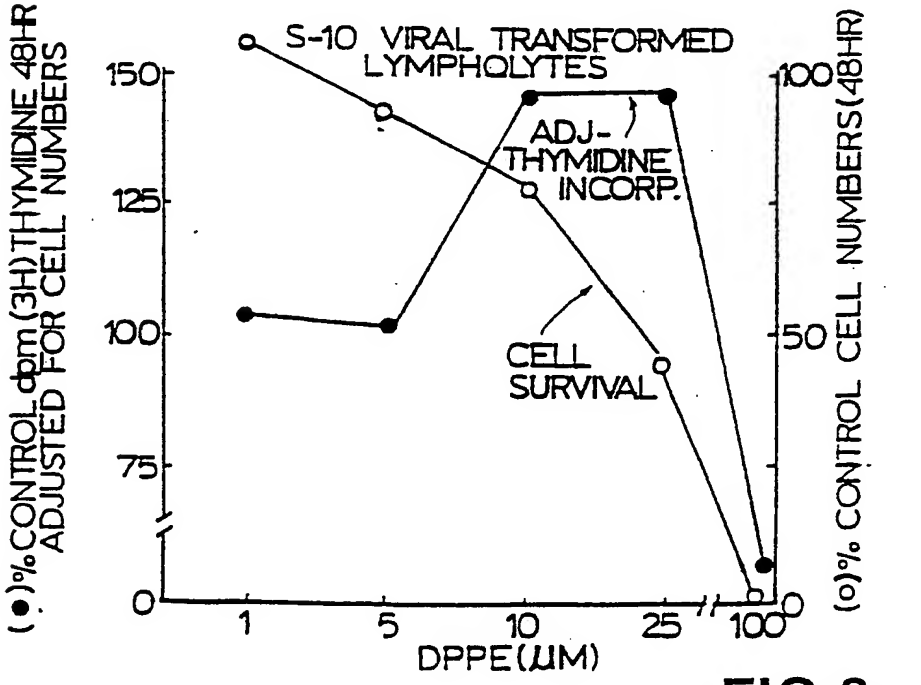


FIG.3.

SUBSTITUTE SHEET

FIG.2.

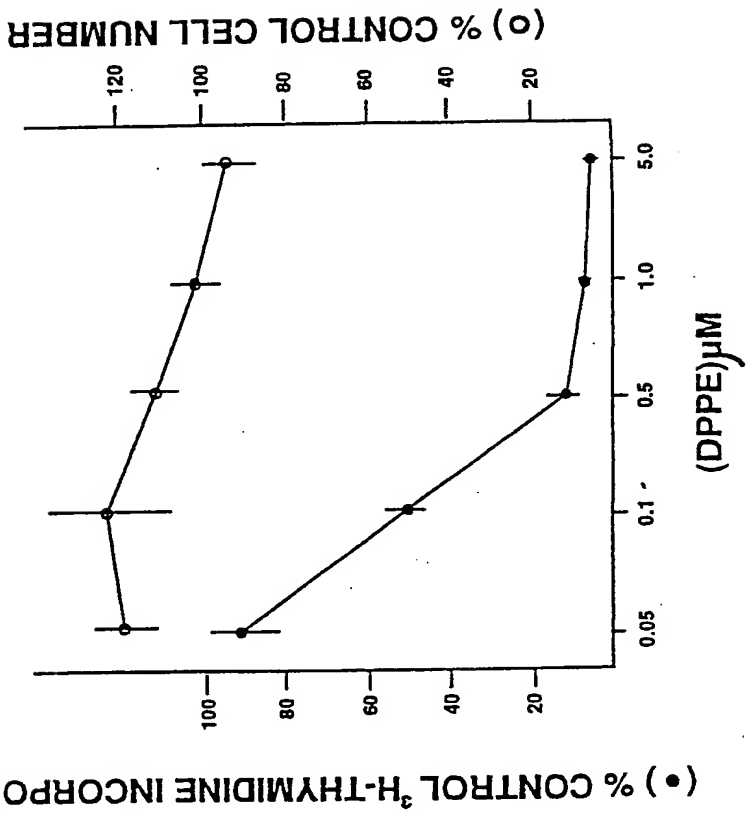

(o) % CONTROL CELL NUMBER
120
100
80
60
40
20
5.0
1.0
0.5
0.1
0.05
(DPPE)μM
100
80
60
40
20
(•) % CONTROL ^{3}H-THYMIDINE INCORPORATION

FIG. 4.

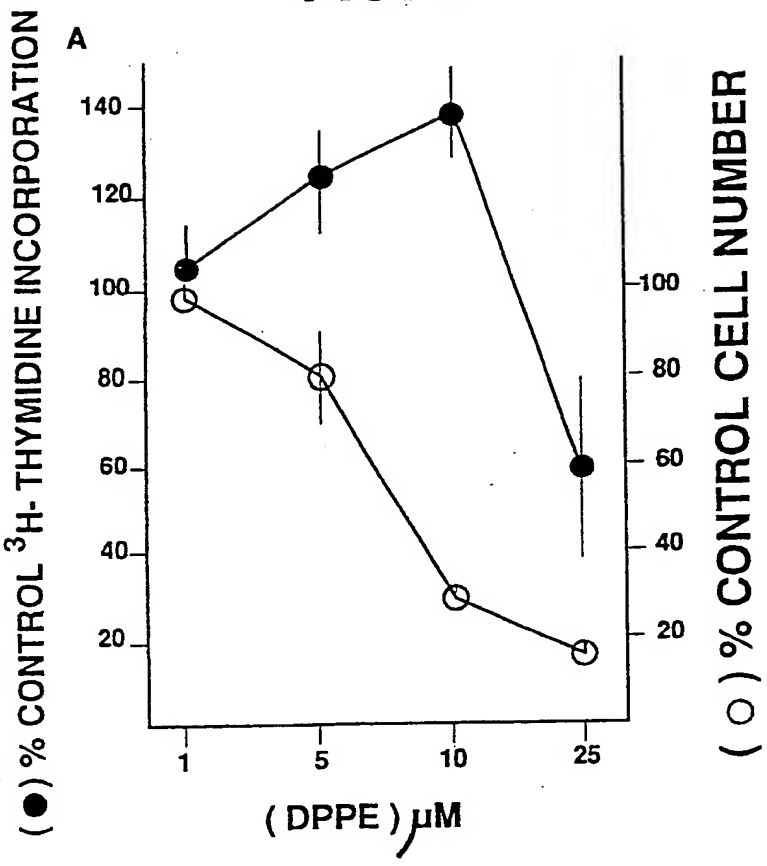
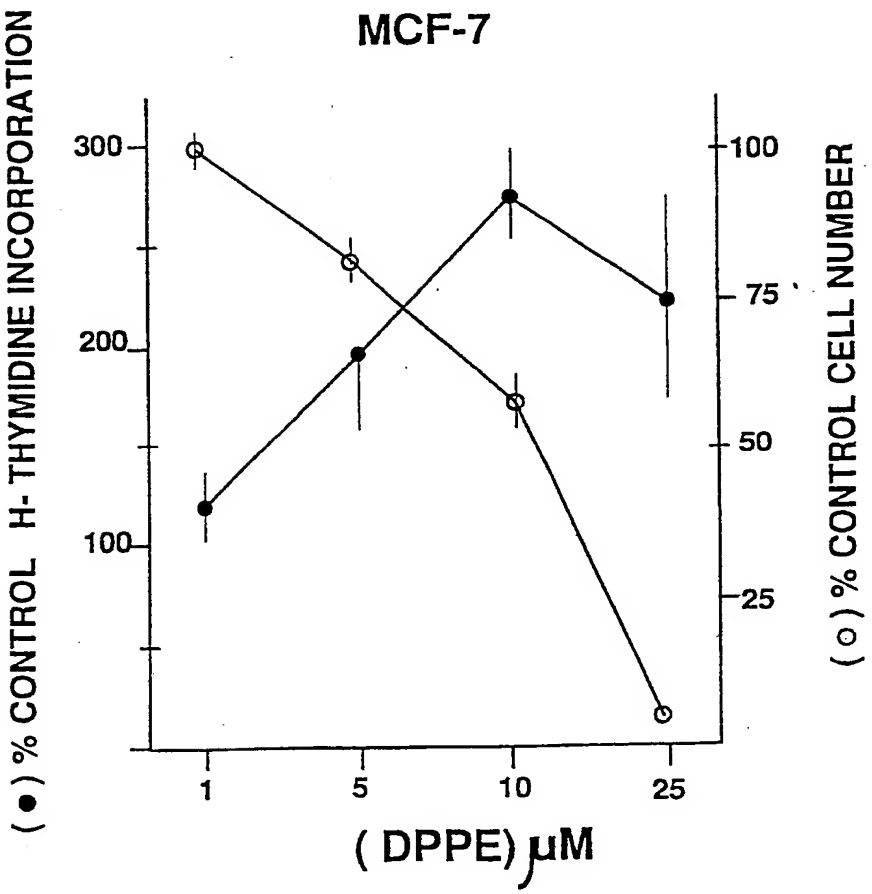

A
(●) % CONTROL ^{3}H- THYMIDINE INCORPORATION
140
120
100
80
60
40
20
1
5
10
25
(DPPE) μM
100
80
60
40
20
(○) % CONTROL CELL NUMBER

FIG.5.


MCF-7
(●) % CONTROL H- THYMIDINE INCORPORATION
300
200
100
100
75
50
25
(○) % CONTROL CELL NUMBER
1
5
10
25
(DPPE) μM

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 91/00449

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 45/08, 31/13, 31/535, 47/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP, A1, 0153160 (BRANDES, LORNE J.) 28 August 1985, see the whole document	21-28
A	Dialog Information Services, File 155, MEDLINE, Dialog accession no.05533338, Medline accession no. 85149338, Brandes LJ et al: "Evidence that the anti-estrogen binding site is a histamine or histamine-like receptor", Biochem Biophys Res Commun Jan 31 1985, 126 (2) p 905-10	21-28
A	Dialog Information Services, File 155, MEDLINE 66-92 May, Dialog accession no. 07418183, Medline accession no. 90325183, Brandes LJ et al: "Study of the in-vivo antioestrogenic action of N,N-diethyl-2-4-(phenylmethyl)phenoxyethanamine HC1 (DPPE), a novel intracellular histamine antagonist and antioestrogen binding site ligand", J Reprod Fertil May 1990, 89 (1) p 59-67	21-28

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7th April 1992	12. 05. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Dialog Information Services, File 159, Cancerlit 63-92 Mar, Dialog accession no. 00606997, 87629197, MEDL/87273175, Brandes LJ et al: "Histamine and growth: interaction of antiestrogen binding site ligands with a novel histamine site that may be associated with calcium channels", Cancer Res; 47(15): 4025-31 1987	21-28

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 1-20, because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/CA 91/00449

SA 54246

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/02/92
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0153160	28/08/85	CA-A-	1229604	24/11/87
		JP-A-	60190742	28/09/85
		US-A-	4803227	07/02/89

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

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Vol. 154, 247–250, July 1995
Printed in U.S.A.

GROWTH-INHIBITORY EFFECTS OF SEROTONIN UPTAKE INHIBITORS ON HUMAN PROSTATE CARCINOMA CELL LINES

MANSOOR ABDUL, CHRISTOPHER J. LOGOTHETIS AND NASEEMA M. HOOSEIN*

From the Department of Genitourinary Medical Oncology, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Growth stimulation of a variety of cell types by the neurotransmitter serotonin has been reported. We have examined the effects of three serotonin-uptake inhibitors, 6-nitroquipazine, zimelidine and fluoxetine (Prozac, Eli Lilly Co., Indianapolis, Indiana) on human prostate carcinoma cell lines. In vitro, all 3 of these compounds inhibited the proliferation of PC-3, DU-145 and LNCaP cells in a dose-dependent manner. Also, all 3 compounds blocked the uptake of a radiolabeled analog of serotonin by the prostate carcinoma cell lines. The order of potency for inhibition of growth as well as for serotonin uptake was fluoxetine > zimelidine > 6-nitroquipazine. The growth of subcutaneous, PC-3 xenografts in athymic nude mice was significantly inhibited by fluoxetine. These results implicate biogenic amines such as serotonin in the growth of prostate carcinoma cells and indicate the potential use of serotonin-uptake inhibitors for the treatment of prostate cancer.

KEY WORDS: prostatic neoplasms, serotonin uptake inhibitors, growth inhibitors

Growth stimulatory effects of the neurotransmitter serotonin[1] have been described on a variety of cell types including vascular smooth muscle cells,[2] lung fibroblasts,[1] renal mesangial cells,[3] normal as well as transformed intestinal epithelial cells,[4] pancreatic carcinoid cells[5] and small cell lung carcinoma.[6] Serotonin is produced and contained in several tissues, particularly the central nervous system[7] and blood platelets.[8] Serotonin and its metabolites are present in the normal[9] and hyperplastic[10] human prostate, and it is a marker of neuroendocrine differentiation in prostatic carcinoma.[11] We have reported the presence of serotonin binding sites and antiproliferative effects of the 5-HT_{1A} antagonist pindobind on prostate carcinoma cell lines.[12] In this report, we describe the effects of serotonin uptake inhibitors on the growth of 3 human prostatic carcinoma cell lines, androgen-insensitive PC-3 and DU-145 cells and androgen-sensitive LNCaP cells.

MATERIALS AND METHODS

Materials. PC-3 cells were obtained from the American Type Culture Collection (Rockville, Maryland), and DU-145 cells were provided by Dr. Don Mickey (University of North Carolina, Chapel Hill, North Carolina). LNCaP cells used at passages 40–45 were supplied by Dr. Gary Miller (University of Colorado, Denver, Colorado). Cells were maintained in Leibovitz's L-medium containing 5% fetal bovine serum and antibiotics. Serotonin uptake inhibitors 6-nitroquipazine and zimelidine dihydrochloride were purchased from Research Biochemicals, Inc. (Natick, Massachusetts). Fluoxetine hydrochloride was provided by Eli Lilly and Co. (Indianapolis, Indiana). ^{3}H-labeled 8-OH DPAT ([8-hydroxy-2-[di-n-propyl-amino]tetralin) and thymidine were purchased from Amersham (Arlington Heights, Illinois).

In vitro proliferation. Cells were plated in serum-supplemented growth medium at 10,000 cells per well in 96-well plates. The following day cells were treated with serotonin uptake inhibitors at the indicated concentrations in serum-free Leibovitz's medium containing phenol red. No androgens were added. After 3 days, cell numbers were determined using the MTT assay as previously described.[13, 14] Each experiment was repeated at least 4 times.

In vivo growth. Five-week-old male BALB/c athymic nude mice (Harlan Sprague-Dawley, Indianapolis, Indiana) were injected subcutaneously with 2 million PC-3 cells. Four weeks later (day 0) approximately 60% of the animals formed tumors. Animals with tumors (average volume of about 75 mm.^{3}) were randomly divided into 2 groups of 6 animals each and treated once daily with fluoxetine HCl (40 μg. per day administered subcutaneously) for 6 weeks. Tumors were measured with microcalipers each week, and tumor volume was calculated by the following formula: length × width × height × 0.5236.

Serotonin uptake assay. DU-145 cells were grown to near confluency in 12-well plates (Falcon, Lincoln Park, New Jersey) in serum-supplemented growth medium. Cells were then maintained in serum-free growth medium for 48 hours. Prior to the uptake assay, cells were preincubated in serum-free growth medium containing monamine oxidase (MAO) inhibitor Ro 41-1049 (3 μM.) for 45 minutes to prevent serotonin analog metabolism via monamine oxidase. Uptake was initiated by removal of medium containing MAO inhibitor and addition of growth medium containing 8.8 nM. ^{3}H-8-OH DPAT (specific activity 228 Ci/mmol.) and cold serotonin uptake inhibitors at the indicated concentrations. After incubation for 1 hour at 37C cells were rapidly washed 3 times with 1 ml. of growth medium containing 1 μM. 6-nitroquipazine, which was included to prevent efflux of the serotonin analog. Cells were harvested by shaking in 1 ml. of 2N NaOH for 20 minutes and were counted in a scintillation counter. Under these conditions total uptake of the radiolabeled serotonin analog was 65.5 pmol./10^6 DU-145 cells. Nonspecific uptake of radioactivity determined in the presence of a 10,000-fold molar excess of cold fluoxetine was 5.3% of total uptake.

Student's t test was used for data analysis.

RESULTS

Concentration-dependent inhibition of all 3 prostate carcinoma cell lines by 6-nitroquipazine (fig. 1, A), zimelidine (fig. 1, B) and fluoxetine (fig. 1, C) was observed in vitro. The half-maximal concentrations required for growth inhibition

Accepted for publication January 3, 1995.
* Requests for reprints: Department of Genitourinary Medical Oncology, Box 155, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030.
This work was supported by N.I.H. Grant CA63225 to N.M.H. and CaP CURE Award (Santa Monica, California) to C.J.L.

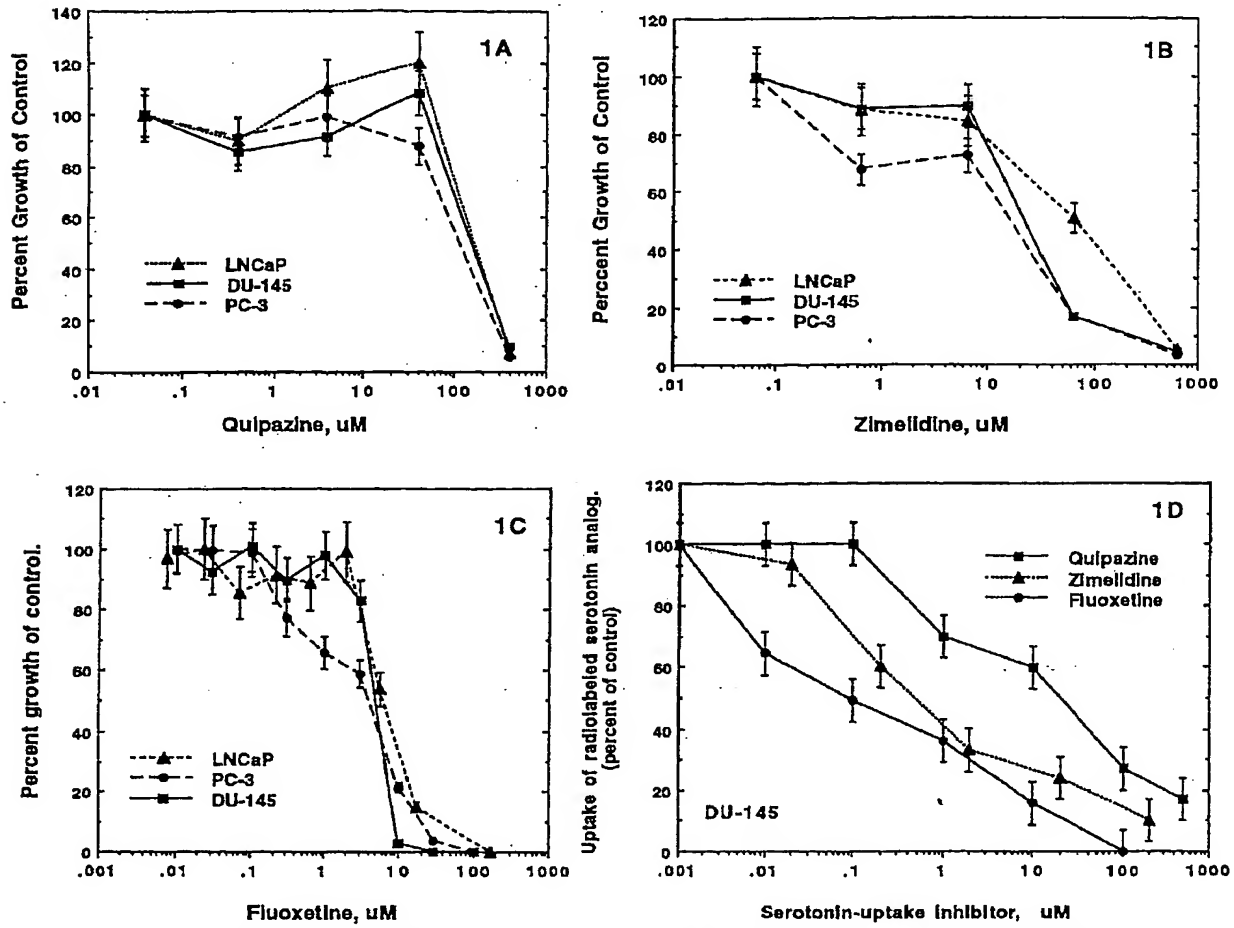

FIG. 1. Growth inhibitory effects of serotonin-uptake inhibitors, 6-nitroquipazine (1A), zimelidine (1B) and fluoxetine (1C) on in vitro growth of 3 human prostate tumor cell lines, PC-3, DU-145 and LNCaP. Inhibition of uptake of radiolabeled serotonin analog by 3 serotonin-uptake inhibitors in DU-145 cells (1D).

of the prostate tumor cell lines by the 3 serotonin uptake inhibitors are indicated in the table. In the case of each inhibitor, the IC_{50} value was slightly higher for the androgen-responsive LNCaP cell line than for the androgen-unresponsive DU-145 and PC-3 cell lines (fig. 1, A–C, table). Fluoxetine was the most potent growth inhibitor of all 3 cell lines (fig. 1, table). Growth curves with PC-3 cells indicated that fluoxetine had a cytostatic effect at a concentration of 10 μM. but cytotoxic effects at concentrations higher than 10 μM. (fig. 2).

Of the 3 serotonin-uptake inhibitors tested, fluoxetine was the most potent inhibitor of the uptake of a radiolabeled serotonin analog by DU-145 cells (fig. 1, D). Similar results were obtained in the serotonin-uptake assay with LNCaP and PC-3 cells (data not shown). In DU-145 cells, with each serotonin-uptake inhibitor the IC_{50} value for growth inhibition was approximately equal to the IC_{75} value for inhibition of serotonin uptake (fig. 1, table).

Summary of in vitro data for growth inhibition as well as serotonin-uptake

Serotonin uptake inhibitor	Growth inhibition IC_{50} (μM.)			Inhibition of serotonin uptake (DU-145)	
	PC-3	DU-145	LNCaP	IC_{50} (μM.)	IC_{75} (μM.)
1. Fluoxetine	4.0	5.0	6.0	0.1	3.6
2. Zimelidine	15.0	25.0	65.0	0.5	20.0
3. 6-nitroquipazine	110.0	140.0	150.0	11.0	120.0

The tumorigenic PC-3 cell line was used to evaluate the effect of fluoxetine on the growth of prostate carcinoma cells in vivo in immunodeficient mice. Results depicted in figure 3 indicate a significant reduction in the growth rate of subcutaneous PC-3 xenografts in athymic nude mice treated with fluoxetine at a dose of 40 μg. per day (2 mg./kg./day). After 6 weeks of treatment with fluoxetine at this dose no overt toxicity or weight loss was observed in the animals. The Prozac package insert indicates that after 30 days of dosing at 40 mg. per day, plasma concentrations of fluoxetine of 91–302 ng./ml. (0.3 to 0.9 μM.) have been observed. At 140 mg. per day (equivalent to 2 mg./kg./day in mice) higher plasma concentrations could be achieved. Due to subcutaneous injection of fluoxetine near the tumor site, concentrations of 5 to 10 μM. may have been attained. Since cytotoxic effects were not observed in the in vivo experiment (fig. 3), it is unlikely that local concentrations above 10 μM. were achieved.

DISCUSSION

Serotonin has numerous physiological functions. This monoamine has been implicated in the regulation of circadian rhythmicity, sleep-wakefulness, sexual behavior, mood changes, appetite and pain perception.[15] The pharmacology

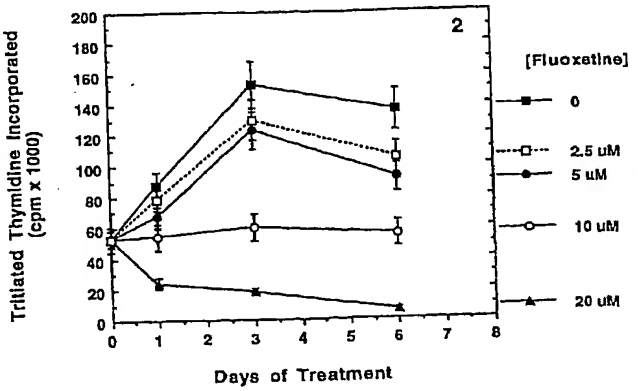

FIG. 2. Retardation of growth of PC-3 cells in vitro. 10,000 cells per well were plated in 96-well plates with 5% fetal bovine serum-supplemented Leibovitz's medium. Tritiated thymidine was then added at 1 μCi. per well. After 24 hours on Day 0, cells were treated with indicated concentrations of fluoxetine in serum-free Leibovitz's medium. Thymidine incorporation was determined on days 0, 1, 3 and 6. Values shown are mean ± SD of 6 assay points each. This experiment was repeated an additional time with similar results.

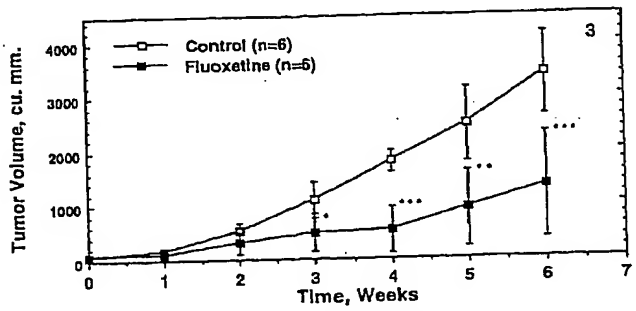

FIG. 3. Retardation of growth of PC-3 cells in vivo, in athymic nude mice by fluoxetine. There were 6 animals in each of 2 groups. Values shown are mean ± SD of 6 tumors, from single experiment. * $p < 0.025$, ** $p < 0.01$, *** $p < 0.005$ (Student's t test).

of serotonin is highly developed.[15,16] Serotonin analogs are currently used in the treatment of numerous disorders, including depression, anorexia, anxiety, migraine and chemotherapy-induced emesis.[15] Serotonin is increasingly being recognized as a growth factor of normal as well as transformed cells.[1-8,12] Similarly to our results with prostate carcinoma cells, serotonin has been implicated in the growth of colon tumors.[4,17] Also, serotonin has recently been shown to promote the growth of small cell lung carcinoma in an autocrine manner.[6]

The serotonin uptake inhibitor fluoxetine has been reported to enhance the growth of transplantable C-3 fibrosarcoma and B16F10 melanoma in mice as well as dimethylbenzanthracene-induced mammary tumors in rats.[18] These findings of tumor promotion by fluoxetine are contradictory to those obtained after extensive testing of fluoxetine for carcinogenicity in rodents.[19] At doses 1 to 25 times the equivalent daily human dose for depression (20 mg. per day), there was no evidence of an increased incidence of any type of unusual or commonly occurring spontaneous neoplasm in either rats or mice used in the study.[19] In addition, significant decreases in the incidences of pituitary adenomas in male and female rats and mammary adenomas and fibrosarcomas in female rats were found. Also, Tutton and Barkla[20] have reported that fluoxetine and another serotonin uptake inhibitor, citalopram, suppress cell division in dimethylhydrazine-induced colonic tumors in rats and retard the growth of 2 human colon tumors propagated in immune-deprived mice. Our results of growth retardation of human prostate tumor cells by serotonin uptake inhibitors in vitro (fig. 1) and in vivo in immune-deficient mice (fig. 2) are in agreement with the findings of Tutton and Barkla[20] who have suggested that serotonin uptake inhibitors should be studied as antineoplastic agents in humans.

The IC_{50} values for growth inhibition by the serotonin uptake inhibitors were not significantly different in the 3 cell lines, although those for PC-3 cells were the lowest in the case of each amine-uptake blocker tested (table). Also, the levels of radiolabeled serotonin analog uptake were similar in LNCaP and DU-145 cells, but 4-fold higher in PC-3 cells (data not shown). These results suggest a greater role of biological amines in the growth of PC-3 cells, which display an aggressive growth behavior in vivo in immune-deprived mice, than in the growth of the relatively indolent LNCaP and DU-145 cells. Treatment of LNCaP cells with 3 nM. dihydrotestosterone did not affect the level of serotonin-uptake by this androgen-sensitive cell line. The order of potency of the 3 uptake blockers for growth inhibition (fluoxetine > zimelidine > 6-nitroquipazine) was the same as that for blocking amine uptake (table). In addition, the IC_{50} for growth inhibition matched the IC_{75} for uptake inhibition for all 3 serotonin uptake blockers in DU-145 cells (table). These data suggest that the growth suppressive effects observed are not nonspecific but are related to inhibition of amine uptake.

In the animal model, significant growth inhibition of PC-3 cells was observed at a dose of 2 mg./kg./day for 4 to 6 weeks (fig. 3). This is equivalent to a human dose of 140 mg. per day. The Prozac package insert suggests that no therapeutic advantage is gained by exceeding the dose of 80 mg. per day. Nonetheless, Prozac has been used for the treatment of psychiatric disorders for extended periods of time at doses greater than 100 mg. per day without significant adverse effects.[21] Mild, reversible hepatic toxicity and an increase in the frequency of agitation (mania) have been reported.[21] Although a significant increase in permanent toxicity was not found, the effectiveness of such an increased dose in psychiatric disorders is not of proved benefit. Moreover, patients who take excessive Prozac acutely (intentionally or by error) may suffer an increased risk of agitation or seizure but do not suffer fatal or permanently debilitating side effects.[21] Hence, there is clinical evidence to support the safety of a dose of 140 mg. per day. On the basis of this clinical information we believe that the predicted therapeutically active dose of fluoxetine (140 mg. per day) can be used for treatment with acceptable side effects.

Inhibition of androgen-dependent growth of prostatic carcinoma by androgen withdrawal (surgical or medical castration) has resulted in significant palliative benefit. Unfortunately, alternate pathways of sustaining growth result in clinical relapse. Neuroendocrine differentiation in prostatic carcinomas, which may occur with a frequency as high as 50%, has been associated with androgen-independent growth.[11] Most if not all prostatic neuroendocrine cells contain serotonin.[11] Our results suggest that serotonin promotes the growth of prostate tumors and could be a target for therapy.

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Human Keratinocyte Cell Lines Differ in the Expression of the Collagenolytic Matrix Metalloproteinases-1, -8, and -13 and of TIMP-1

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We investigated cells and conditioned media of the three human keratinocyte cell lines HaCaT (non-tumorigenic), A5 (benign, tumorigenic) and II-4RT (malignant, tumorigenic) with regard to production and secretion of the collagenases-1 to -3 (MMP-1, MMP-8 and MMP-13) and TIMP-1 using semi-nested RT-PCR, Western blots, ELISA, immunocytochemistry and casein zymography.

Transcripts of MMP-1, -8, -13 and TIMP-1 were detected in all cell lines by RT-PCR and the corresponding proteins were found in the cytoplasm of all three cell lines by Western blot analysis and/or immunocytochemistry. The conditioned media of the malignant II-4RT cells contain significantly more MMP-1 and MMP-8 than those of HaCaT or A5 as evidenced by immunoblotting and ELISA. In addition to the presence of latent MMP-1, zymography also detected the active form of this enzyme. TIMP-1 was found only in extracts of all three cell lines, predominantly in A5.

This study clearly indicates that the epithelial tumor cells synthesize different collagenases and TIMP-1. The malignant clone secretes increased amounts of distinct collagenases compared to the non-tumorigenic cell line, thereby verifying a correlation between biological behaviour and the amount of collagenases. In addition, we provide clear evidence that MMP-8 is not exclusively found in polymorphonuclear granulocytes, but also in keratinocyte cell lines.

Key words: Collagenase / HaCaT cells / *In vitro* study / Keratinocyte cell lines / MMP / Tumorigenicity.

Introduction

The process of tumor invasion requires degradation of the extracellular matrix by proteolytic enzymes (Matrisian 1992; Birkedal-Hansen *et al.*, 1993). Therefore, the balance between production, activation and inhibition of these enzymes is fundamental for the steps involved in the tumor-associated matrix turnover (Schmitt *et al.*, 1992; Mignatti and Rifkin, 1993; Johnsen *et al.*, 1998). In particular, an increased expression of matrix metalloproteinases (MMPs) has been shown to be involved in tumor invasion and metastasis (Basset *et al.*, 1997; Johnsen *et al.*, 1998).

Until now more than 17 members of the family of zinc-dependent MMPs have been identified (Woessner, 1998). Due to their substrate specifity and structure, MMPs can be classified into collagenases, stromelysins, gelatinases, membrane-type MMPs and other MMPs (Kähäri and Saarialho-Kere, 1997). During tumor invasion, collagenases are particulary important as these enzymes are able to degrade the collagenous connective tissue which represents the bulk of the interstitial matrix.

The three mammalian collagenases known, *i. e.* the fibroblast-type interstitial collagenase, the neutrophil collagenase, and collagenase-3 differ in their substrate preferences. The fibroblast-type interstitial collagenase (MMP-1, collagenase-1; Goldberg *et al.*, 1986) prefers type III collagen over type I, II, VII and X (Gadher *et al.*, 1989; Seltzer *et al.*, 1989). It is widely expressed in various cell types including fibroblasts, chondrocytes, macrophages, endothelial cells and keratinocytes (Freije *et al.*, 1994). Increased levels of this enzyme have been detected in various carcinomas, *e. g.* thyroid and gastric cancer (Kameyama, 1996, Sakurai *et al.*, 1997a, b).

The neutrophil collagenase (MMP-8, collagenase-2; Hasty *et al.*, 1987) preferentially cleaves type I and II collagens, but also type III collagen. This enzyme was thought to be synthesized exclusively by polymorphonuclear neutrophils (PMN) where it is stored as a latent proenzyme in the specific granules (Murphy *et al.*, 1977; Mainardi *et al.*, 1991). However, *in vivo* MMP-8 expression has recently been observed in mononuclear fibroblast-like cells in the rheumatoid synovial membrane, and MMP-8 mRNA was detected in cultured rheumatoid synovial fibroblasts, hu-

man endothelial cells and human chondrocytes (Chubinskaya *et al.*, 1996; Cole *et al.*, 1996; Hanemaaijer *et al.*, 1997).

The collagenase-3 (MMP-13; Freije *et al.*, 1994) shows an exceptionally wide substrate specifity compared to other MMPs. In addition to fibrillar type I, II and III collagens it also degrades type IV, IX, X and XIV collagen, gelatin, tenascin, fibronectin and proteoglycan coreproteins (Knäuper *et al.*,1996; Mitchell *et al.*,1996). Collagenase-3 is expressed by different malignant tumors including breast carcinoma (Freije *et al.*, 1994) and squamous cell carcinoma of the skin, head and neck (Airola *et al.*, 1997; Johansson *et al.*, 1997a; Cazorla *et al.*, 1998).

Activation of MMPs is controlled by specific inhibitors, termed 'tissue inhibitors of metalloproteinases' (TIMPs). So far 4 TIMPs have been characterized which differ in their inhibitory activities for the various MMPs. Thus, TIMP-1 predominantly inhibits MMP-1, -3 and -9 (Mackay *et al.*, 1992), while TIMP-2 preferentially binds to MMP-2 (Goldberg *et al.*, 1989). Besides these inhibitory effects, TIMPs exert other biologically relevant functions and even appear to be involved in the activation of certain MMPs.

So far it is unclear to what extent epithelial tumor cells and the neighbouring stromal cells are responsible for the elevated levels of collagenases in cancer tissues. In order to elucidate the contribution of the tumor cells, we investigated whether the differences in tumorigenicity in a model system of three closely related human keratinocyte cell lines (Boukamp *et al.*, 1988, 1990) are reflected by their profiles of constitutively expressed collagenases.

The cell lines investigated were HaCaT, a spontaneously immortalized human keratinocyte cell line (Boukamp *et al.*, 1988), and the two clones A5 and II-4RT which were derived from HaCaT by stable transfection with the cellular Ha-*ras* oncogene (Boukamp *et al.*, 1990). When implanted into athymic mice HaCaT cells are non-tumorigenic, A5 cells form benign cysts and II-4RT cells develop locally invasive squamous cell carcinomas. Thus the three cell lines represent a unique model of closely related cells with defined *in vivo* tumorigenicity.

Our approach is based on recent observations that in this model the amounts of gelatinases (MMP-2, -9) and stromelysins (MMP-3, -7, -10) synthesized and secreted increase with tumorigenicity, thus providing a potential link between these enzymes and the cellular behaviour (Bachmeier *et al.*, 1998; 2000).

In the present study we provide evidence that all cell lines express the collagenases MMP-1, -8 and -13 as well as TIMP-1. Thus, MMP-8, the neutrophil collagenase, is not specific for leukocytes, but can also be expressed by epithelial tumor cells. The locally invasive cell line secretes increased amounts of MMP-1 and -8; furthermore, in addition to the latent enzyme also active MMP-1 is present in the media. These observations suggest that the collagenases expressed by epithelial tumor cells may directly contribute to invasive tumor growth. In addition, the presence of several collagenases indicates the redundancy of this proteolytic system.

Results

Detection of mRNAs of Collagenases and TIMP-1

Analysis of total RNA by semi-nested RT-PCR demonstrated that the three cell lines express MMP-1 (Figure 1, lanes 1 and 2), MMP-8 (lanes 3 and 4), MMP-13 (lanes 5 and 6) and TIMP-1 (lanes 7 and 8). Primers were chosen so that the PCR amplicons included exon-exon transitions (see Table 1) and thus amplification of genomic DNA would result in products easily recognizable by their size. In addition, the identity of the amplicons was verified by digestion with restriction endonucleases (Table 1, Figure 1).

Detection of Collagenases and TIMP-1 in Cells and Cell Extracts

Protein expression of MMP-1, -8, -13 and TIMP-1 was detected in extracts and conditioned media (see section below) of all three cell lines by means of Western blots, immunohistochemistry, ELISA, and zymography; the high

Table 1 Primers Used for PCR Amplification.

Primer	Sequence	Location
MMP-1 forward	5′-AGATGTGGAGTGCCTGATGT-3′	exon 2
MMP-1 reverse (outer)	5′-CCTGCAGTTGAACCAGCTAT-3′	exon 9
MMP-1 reverse (inner)	5′-GTGCGCATGTAGAATCTGTC-3′	exon 7
MMP-8 forward	5′-ACCAATTACCAAGCAACCAG-3′	exon 2
MMP-8 reverse (outer)	5′-GGGATACATCAAGGCACCAG-3′	exon 5
MMP-8 reverse (inner)	5′-GAGCAGCAACAAGAAACAAG-3′	exon 5
MMP-13 forward	5′-CCAACCCTAAACATCCAAAAAC-3′	exon 6
MMP-13 reverse (outer)	5′-CACCACAAAATGGAATTTGCTG-3′	exon 9
MMP-13 reverse (inner)	5′-GGCATGACGCGAACAATAC-3′	exon 9
TIMP-1 forward	5′-CCAGAGAGACACCAGAGAAC-3′	exon 1/2
TIMP-1 reverse (outer)	5′-GAGGTAAGTGCCATGGTGAG-3′	exon 2
TIMP-1 reverse (inner)	5′-ACTCACCGAGGTCGGAATTG-3′	exon 2

All primers were designed using the GenBank cDNA sequences for MMP-1 (accession number U78045), MMP-8 (accession number J05556), MMP-13 (accession number X75308), and TIMP-1 (accession number Y09720).

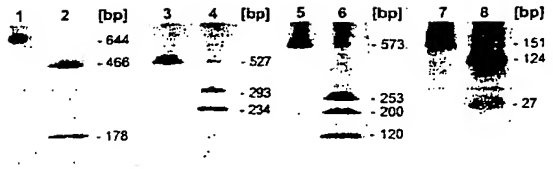

Fig. 1 Detection of MMP-1, -8, -13, and TIMP-1 mRNA by Semi-Nested RT-PCR.
Data are shown for MMP-1 (lanes 1 and 2), MMP-8 (lanes 3 and 4), MMP-13 (lanes 5 and 6) and TIMP-1 (lanes 7 and 8). Reverse transcription and semi-nested PCR were performed with total RNA from about 10^6 cells. Aliquots of the PCR mixtures before (lanes 1, 3, 5 and 7) and after digestion with restriction endonucleases (lanes 2, 4, 6 and 8) were subjected to PAGE. The endonucleases *Sty*I, *Fok*I, *Dde*I and *Hae*III were used to digest amplicons from MMP-1, -8, -13, and TIMP-1, respectively. The gels shown are the results from HaCaT cells, representatively for all three cell lines.

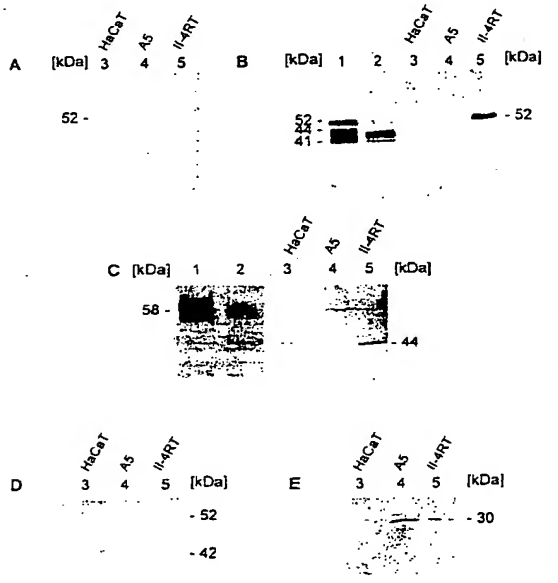

Fig. 2 Western Blot Analyses of Cell Extracts and Serum-Free Conditioned Media of HaCaT, A5 and II-4RT Cells Using Antibodies Specific for MMP-1, -8, -13, and TIMP-1.
Extracts (60 µg protein) analyzed for MMP-1 (A), MMP-13 (D), and TIMP-1 (E). Conditioned Media (15 µg protein) analyzed for MMP-1 (B), and MMP-8 (C). HaCaT cells: lanes 3; A5 cells: lanes 4; II-4RT cells: lanes 5; MMP-1 and -8 controls (B, C): lanes 1: without APMA; lanes 2: after APMA treatment.

sensitivity of zymography and immunohistochemistry allowed to detect distinct proteins where the limits of the Western blot technique were already reached.

Western Blots MMP-1 was detected as faint bands of latent enzyme (52 kDa, Figure 2A) and MMP-13 as bands of latent (52 kDa, Figure 2D) as well as active enzyme (42 kDa, Figure 2D). About equal amounts were found in extracts of all three cell lines. MMP-8 was not detected in the extracts of any cell line by immunoblotting.

In addition, bands of TIMP-1 at ~ 30 kDa were detectable in extracts of all three cell lines. The intensity of the TIMP-1 band was highest in benign A5 cell extracts (Figure 2E; lane 3), lower in those of malignant II-4RT (Figure 2E; lane 4) and hardly detectable in those of HaCaT (Figure 2E; lane 1) (see also Table 2).

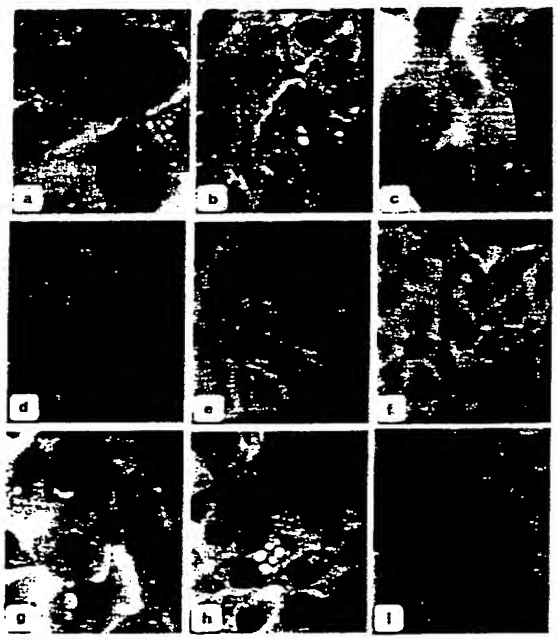

Fig. 3 Immunocytochemical Staining of MMP-1, MMP-8 and MMP-13.
In all three cell lines a cytoplasmic positive staining was seen for MMP-1 [HaCaT, (a); A5, (d); II-4RT, (g)] MMP-8 [HaCaT, (b); A5, (e); II-4RT, (h)] and MMP-13 [HaCaT, (c); A5, (f); II-4RT, (i)]. Magnification 340 x.

Table 2 Detection of MMP-1, -8, -13 and TIMP-1 in Extracts and Conditioned Media on Western Blots.

	Extracts				Conditioned media			
	HaCaT	A5	II-4RT	Form	HaCaT	A5	II-4RT	Form
MMP-1	+	+	+	latent	+	–	+++	latent
MMP-8	–	–	–	–	++	(+)	+++	active
MMP-13	+	+	+	latent	–	–	–	–
	+	+	+	active				
TIMP-1	+	+++	++	–	–	–	–	–

The intensities of the bands were scored from +++ (strong signal) to – (no signal).

Table 3 Scoring of the Immunocytochemical Staining.

	HaCaT	A5	II-4RT
MMP-1	12	12	12
MMP-8	3	6	7.5
MMP-13	8	8	8

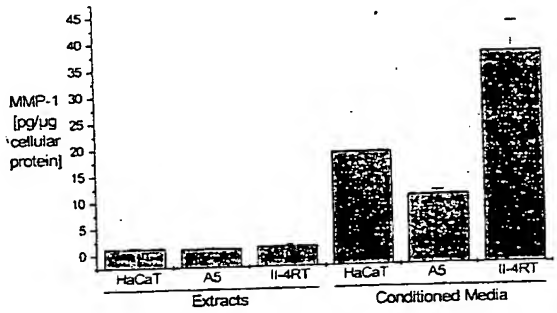

Fig. 4 Quantification of MMP-1 in Extracts and Conditioned Media of HaCaT, A5, and II-4RT Cells.
MMP-1 was quantified using the Amersham Biotrak MMP-1 ELISA kit. Values represent mean ± SD.

Immunocytochemistry The immunocytochemical analysis revealed the presence of immunoreactive MMP-1, -8 and -13 in all three cell lines, although to a different degree. The enzymes were localized intracytoplasmatically in a granular pattern. There was no accentuated membrane staining. Using a scoring system (see also Table 3) for the evaluation of the amounts of protein, we found equally intense stainings for MMP-1 and -13 in HaCaT (Figure 3a, g), A5 (Figure 3b, h) and II-4RT cells (Figure 3c, i). In contrast, the MMP-8 staining score was lower in HaCaT cells (Figure 3d) compared to the two tumorigenic cell clones – the benign A5 cells (Figure 3e) and the malignant II-4RT cells (Figure 3f) – which showed only minor differences.

ELISA The amounts of MMP-1 protein in the cytoplasm of the three cell lines were quantified by MMP-1-ELISA. As shown in Figure 4 the amounts of MMP-1 protein in HaCaT, A5 and II-4RT were virtually identical (1.3, 1.1, and 1.4 pg/µg of cellular protein, respectively).

Detection of Collagenases and TIMP-1 in Conditioned Media

Western Blot In contrast to the analysis of cytoplasmic collagenases-1 and -2 the pattern of secreted MMP-1 and MMP-8 differed significantly between malignant II-4RT cells and the other two cell lines (Figure 2B and 2C): while MMP-1 could not be found in media of benign A5 cells (Figure 2B, lane 4), it was present in small amounts in those of normal HaCaT cells (Figure 2B, lane 3) and enhanced in media of malignant II-4RT cells (lane 5). Secreted MMP-8 was visible as a 44 kDa band of active MMP-8. It was present in media of HaCaT (Figure 2C, lane 3) and A5 cells (hardly visible in Figure 2C, lane 4) and severalfold elevated in those of malignant II-4RT cells (Figure 2C, lane 5) (see also Table 2).

To verify whether the MMPs detected by Western blotting represent the latent or the active forms, purified MMP-1 and -8 with and without APMA treatment were used as controls. The 52 kDa band of the cell culture supernatants comigrated with the 52 kDa band of the control (MMP-1 without APMA treatment; Figure 2B, lane 1), indicating the presence of latent MMP-1 in the samples. In addition also active MMP-1 (44-41 kDa) was visible in the samples not treated with APMA (Figure 2B, lane 1). The 52 kDa band almost completely disappeared upon treatment of MMP-1 with APMA, so that only the bands of active MMP-1 (44-41 kDa) were visible (Figure 2B, lane 2). Application of MMP-8 controls (latent form: Figure 2C, lane 1; after APMA activation: Figure 2C, lane 2) demonstrated that the 44 kDa band corresponded to the active form of neutrophil collagenase (see also Table 2).

TIMP-1 and MMP-13 were not detectable in the conditioned media of any of the cell lines.

ELISA The enhanced secretion of MMP-1 by the malignant cells was confirmed and quantified by ELISA. As shown in Figure 4, HaCaT cell culture supernatants contained 19 pg/µg, those of benign A5 10.7 pg/µg and of malignant II-4RT 36 pg/µg of secreted protein.

Zymography In addition to the latent form of MMP-1 (52 kDa) the active enzyme (41 kDa) was found in conditioned media of benign A5 (Figure 5, lane 3) and malignant II-4RT (Figure 5, lane 4) cells by means of zymography. Comparison of the band pattern with that of MMP-1 controls clearly demonstrated that the lytic zones at 52 and 41 kDa correspond to the latent and active forms of MMP-1 (Figure 5, lane 1). The amount of active interstitial collagenase is very low and obviously below the detection limit of the less sensitive Western blot technique. In addition to the bands corresponding to MMP-1 also lytic bands at 60 and 56 kDa were obtained for all cell lines (Figure 5) which were not further characterized in this study.

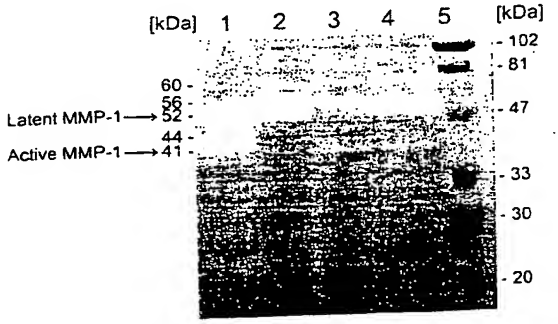

Fig. 5 Casein-Zymography of Conditioned Media.
Lane 1, MMP-1 control; lanes 2 – 4, conditioned media of HaCaT, A5 and II-4RT cells (20 µg protein per lane); lane 5, marker proteins.

Discussion

The dissolution of the extracellular matrix is a prerequisite for invasive malignant growth. Recent studies provide a large body of evidence that particularly matrix metalloproteinases (MMPs) are involved in this tissue degradation. Based on their structures and substrate specificities the MMPs have been classified into membrane-bound MMPs (MT-MMPs), which are mainly involved in the activation of non-bound MMPs, gelatinases (MMP-2, -9), which degrade non-helical collagen and non-collagenous proteins, stromelysins (MMP-3, -7, -10, -11), which mainly dissolve glycoproteins, and collagenases (MMP-1, -8, -13), which are the only mammalian enzymes that are able to cleave fibrillar collagens.

This substrate diversity is assumed to have major biological implications, particularly during the invasive growth of malignant tumors. Thus, the cleavage of the basement membrane as the first barrier for malignant epithelial cells by gelatinases and stromelysins needs to be followed by the breakdown of other interstitial collagen by collagenases. The production and activation of collagenases therefore is pivotal for further tumor growth and local tumor expansion. Indeed, various studies provide evidence that *in vivo* an enhanced production of interstitial collagenases is associated with a more aggressive growth potential and poor clinical outcome (Murray *et al.*, 1996, 1998; Cazorla *et al.*, 1998; Airola *et al.*, 1999; McCarthy *et al.*, 1999).

In a previous study (Bachmeier *et al.*, 1998; 2000) we have shown that HaCaT keratinocytes and the derived clonal cell lines of different tumorigenicity represent an interesting *in vitro* model for studying the biological role of the expression and secretion of gelatinases and stromelysins: the malignant cell clone (termed II-4RT, derived from the parental cell line HaCaT by c-Ha-ras oncogene transfection, Boukamp *et al.*, 1990) produces larger amounts of these enzymes than the benign cell line A5 and particulary the parental non-tumorigenic cell line HaCaT. This protease profile correlates well with the biological behaviour of these cell lines that has been extensively characterized by implantation studies in athymic mice: injection of HaCaT cells resulted in rapidly regressing nodules, A5 cells formed slowly expanding cysts without signs of invasive growth, while II-4RT cells produced locally invasive squamous cell carcinomas (Boukamp *et al.*, 1990). Thus, the enhanced production and secretion of gelatinases and to some extend of stromelysins in the tumorigenic clones may result in the degradation of type IV collagen and other components of the basement membrane.

In the present report we extended our studies on this model system to the analysis of the collagenases MMP-1, MMP-8 and MMP-13. These three collagenases are of special interest because they are able to degrade fibrillar collagens in the extracellular space, making them susceptible to further degradation by other MMPs. Thus, secretion of the collagenases enables tumor cells to invade the interstitial matrix.

Using RT-PCR all three cell lines were shown to express the mRNAs of MMP-1, -8, -13 and of TIMP-1, and thus are capable to synthesize the corresponding proteins. On the protein level, MMP-1 was significantly increased in the media of malignant cells when compared to the benign A5 and the non-tumorigenic HaCaT. So far the expression of this enzyme in HaCaT keratinocytes has not been described; however, several authors have reported the presence of MMP-1 in primary cultures of human squamous cell carcinomas (Petersen *et al.*, 1987; Bailly *et al.*, 1990). Interestingly, in addition to the latent form of MMP-1 also the active enzyme was found in the media of tumorigenic cell lines as detected by zymography, indicating that this collagenase may be activated by the tumor cells themselves even without the support of stromal cells. The intracellular levels of MMP-1 were about equal in the three cell lines as shown by ELISA, immunocytochemistry and Western blotting. The higher proportion of MMP-1 in media of the malignant II-4RT cells suggests that production and secretion of MMP-1 in II-4RT cells are enhanced in comparison to A5 and HaCaT cells and are linked to the malignant infiltrative behaviour.

In addition to MMP-1, MMP-8 was detected in all cell lines. The occurrence of this enzyme has so far not been reported in epithelial tumor cell lines, but was found in the granules of neutrophil leukocytes (Murphy *et al.*, 1977; Mainardi *et al.*, 1991) and also in other non-epithelial cells such as rheumatoid synovial fibroblasts, endothelial cells (Hanemaaijer *et al.*, 1997) and human chondrocytes (Chubinskaya *et al.*, 1996; Cole *et al.*, 1996). Cell-associated MMP-8 was identified in all three cell lines by immunocytochemistry, but not by Western blotting, most likely due to the lower sensitivity of this technique. MMP-8 was found on immunoblots of conditioned media of HaCaT and II-4RT; larger amounts are secreted by the malignant II-4RT than by non-tumorigenic HaCaT, while no MMP-8 was seen in media of A5 cells. As MMP-8 is known to hydrolyze native type I collagen with high efficiency (Hanemaaijer *et al.*, 1997), the secretion of this enzyme may be important for the invasive behavior of tumor cells.

MMP-13 was observed exclusively in cellular extracts as evidenced by Western blotting and by immunocytochemistry, but not in conditioned media. This is well in line with the previous report by Johansson *et al.* (1997b) who did not find MMP-13 in the media of unstimulated HaCaT cells; however, they reported that stimulation by TNF-α and TGF-β resulted in a release of the enzyme into the media. *In vivo* studies on various tumors revealed MMP-13 to be expressed by the epithelial tumor cells (Johansson *et al.*, 1997a; Cazorla *et al.*, 1998; Balbin *et al.*, 1999); in these cases the expression of MMP-13 may be due to stimulation by cytokines released *e. g.* by stromal cells.

The overall increased secretion of collagenolytic activity by II-4RT as compared to HaCaT and A5 cells seems to be reflected in the *in vivo* behavior of the cells: while the parental, non-tumorigenic HaCaT and the benign A5 cells apparently do not destroy their surrounding interstitial matrix *in vivo*, the malignant II-4RT cells show local dissolu-

tion of the collagenous matrix (Boukamp *et al.*, 1990) and therefore require collagenolytic activity in order to grow invasively.

Finally, we analyzed the expression of TIMP-1, which is capable to inhibit most MMPs, particularly MMP-1 (Matrisian, 1990). Interestingly, this inhibitor was detected in extracts of all three cell lines but no secretion could be demonstrated. The highest cell-associated levels were found in the tumorigenic, benign A5 cells, intermediate in malignant II-4RT cells, and the lowest in the non-tumorigenic HaCaT cells. Thus the amounts of cellular TIMP do not correlate well with those of the intracellular or secreted MMPs. At present, the implication of these findings, particularly for the control of the collagenases, is unclear. This subject is further complicated by the observation that TIMPs are not only MMP-inhibitors, but are also involved in protease activation (Strongin *et al.*, 1995).

Taken together, the results presented support the concept that the profile of MMPs synthesized and secreted by tumor cells correlates with the invasive phenotype of the respective cells. The increased constitutive secretion of MMP-1 and -8 by the malignant II-4RT cells appears to be an important molecular tool for the degradation of the extracellular matrix. Secretion and subsequent activation of MMP-13 seems to require the interaction with stromal cells, since this collagenase is found only in conditioned media of TNF-α / TGF-β stimulated but not of unstimulated HaCaT keratinocytes (Johansson *et al.*, 1997b). Surprisingly, the cell lines not only synthesize the collagenases MMP-1 and MMP-13, which are well known to be tumor-associated, but also the neutrophil collagenase MMP-8. Finally, the results confirm the conclusion of our accompanying study (Bachmeier *et. al.*, 2000) that the system of the three keratinocyte cell lines can serve as a highly valuable model for investigating correlations between the tumorigenic phenotype of cells and the expression of tumor-associated constituents.

Materials and Methods

Cells and Culture Conditions

Cell Culture Conditions The keratinocyte cell lines HaCaT, A5 and II-4RT (Boukamp *et al.*, 1988, 1990) were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. The cells were grown in DMEM (1 g/l glucose) supplemented with 10 % heat inactivated fetal calf serum, 0.35 mg/ml L-glutamine and 0.05 mg/ml gentamycin sulfate; for A5 and II-4RT cells gentamycine sulfate was substituted by 0.2 mg/ml geneticine. The medium was changed every three days. For subcultures cells were harvested after brief treatment with 0.1 % trypsin/EDTA solution and seeded at a dilution of 1:10. Cells between passages 12 and 55 were used for studies.

Preparation of Serum-Free Conditioned Medium Cells were grown to approx. 90 % confluence in 75 cm^2 plastic culture flasks containing 15 ml medium. The cultures were rinsed three times with Ca^{2+}- and Mg^{2+}-free PBS and subsequently 10 ml of serum-free medium was added. After 2 days, the conditioned medium was collected, concentrated by ultrafiltration (exclusion limit 10 kDa), and stored at −20 °C.

Harvesting of Cells Cells grown to confluence in serum-containing medium were washed three times with 5 ml PBS and harvested by scraping in 5 ml PBS. The cell supensions were centrifuged and the cells washed twice by suspension with 50 ml PBS and centrifugation (10 min at 300 *g*). Aliquots of 1.5 – 1.7×10^7 cells were stored at −70 °C until analysis.

Preparation of Cell Extracts 50 µl lysis buffer (10 mM Na_3PO_4; 0.4 M NaCl; 0.2 % Triton X-100) was added to an aliquot of frozen cells. The mixture was sonified and after centrifugation for 14 min at 15 000 *g* the supernatant containing the soluble proteins was collected and either analyzed immediately or stored at −20 °C.

Determination of Protein Concentration Protein concentrations were determined by the BCA protein assay (Pierce, Oud-Beijerland, Netherlands) with bovine serum albumin as standard.

Zymography

Electrophoresis was carried out under non-reducing conditions. After renaturation of the proteins by incubation of the gels in 25 g/l Triton X-100 at room temperature for 2 × 10 min they were incubated in 50 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl, 0.02 % Brij35 and 10 mM $CaCl_2$ at 37 °C for 18 h. The gels were stained with Coomassie Brilliant Blue R-250. After destaining zones of proteolytic activity became visible as transparent bands in the blue gel.

As controls MMP-1 from human rheumatoid synovial fibroblasts (Calbiochem, La Jolla, USA) and MMP-8 from human neutrophil granulocytes (Calbiochem) were used. Rabbit muscle phosphorylase b, bovine serum albumin, ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, hen egg lysozyme (Bio-Rad prestained markers, low range) were used as molecular mass markers.

Reverse Transcription and Polymerase Chain Reaction

RNA Extraction Total RNA was extracted from cells according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) using TRI Reagent (Sigma, Deisenhofen, Germany). Total RNA was quantified at 260/280 nm. Aliquots were stored in DEPC-treated water or 0.1 M sodium acetate at −80 °C.

Reverse Transcription cDNAs were synthesized from 1 µg of total RNA using the specific outer reverse primers (Table 1). Reactions were carried out with the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Freiburg, Germany) following the manufacturer's instructions.

Semi-Nested PCR The first round of PCR was carried out with 1/10 of the synthesized specific cDNA, 10 pmol of each primer, 40 nmol dNTP, 1 U PANScript polymerase (PAN-Systems), 2.5 µl 10x-buffer [16 mM $(NH_4)_2SO_4$, 50 mM Tris/HCl, 0.1 g/l Tween-20, pH 8.8] in a total volume of 25 µl. The $MgCl_2$ concentration in the reaction mixture was 6 mM for MMP-1, 2 mM for MMP-8 and -13, and 3 mM for TIMP-1.

20 (MMP-1) or 25 (MMP-8, -13 and TIMP-1) cycles were carried out with 30 s denaturation (94 °C), 30 s annealing (56 °C), and 30 s (MMP-8, TIMP-1) or 1 min (MMP-1, MMP-13) synthesis (72 °C).

The second round of PCR with the forward and inner reverse primers was carried out using one µl of the first PCR mixture in a total volume of 50 µl and 35 PCR cycles. Concentrations of all reagents and cycle profiles were as in the first PCR.

Negative controls were carried out in the same manner but without template.

Characterization of RT-PCR Products PCR products were characterized by restriction fragment analyses and size determination using polyacrylamide gel electrophoresis in 14 % gels containing 10 % glycerol.

Immunoblotting

Sixty µg protein of cell extract or 15 µg protein of conditioned media were subjected to SDS-PAGE in 10 % polyacrylamide slab gels under non-reducing conditions. After electrotransfer to a nitrocellulose membrane and blocking with 50 g/l low fat dry milk powder in TTBS (50 mM Tris-base, 150 mM NaCl, pH 7.5, 1 g/l Tween-20) the membranes were incubated for 2 h in polyclonal rabbit antiserum (for anti-MMP-1 see Lichtinghagen *et al.*, 1995; for anti-TIMP-1 see Geisler *et al.*, 1997; anti-MMP-8 and -13 were supplied by Dr. Harald Tschesche, Bielefeld), washed three times for 10 min in TTBS, incubated for 1 h with a 1:1000 dilution of porcine anti-rabbit immunoglobulin G alkaline phosphatase conjugate (DAKO) in 10 g/l low fat dry milk powder in TTBS. After rinsing twice with TTBS and once with TBS (TTBS without Tween), the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium to visualize bands of alkaline phosphatase activity.

Molecular mass marker proteins and purified MMP-1 (150 ng; Calbiochem) and -8 (100 ng; Calbiochem) were used as controls; in some experiments the purified MMPs were activated with APMA prior to electrophoresis.

ELISA

MMP-1 was quantified in extracts and conditioned media from different passages of the three cell lines using a MMP-1-ELISA Kit (Biotrak, Amersham Buchler, Braunschweig, Germany). The antibody used in this ELISA recognizes the zymogen of MMP-1, the active form and the complex with TIMP-1.

Immunocytochemistry

For the immunocytochemical localization of MMP-1, -8 and -13 cells were grown to confluence on silanized sterile glass slides (SuperFrost plus). The medium was discarded, the cells rinsed with Tris buffer, fixed in methanol/acetone (v/v 2:1) for two minutes, and rinsed again. Following incubation with the specific polyclonal primary antibodies (see 'immunoblotting') for 30 minutes (37 °C), rinsing with Tris buffer, and application of the secondary antibody system (Streptavidin-Biotin-Complex method, Hsu and Raine, 1981; DAKO, Hamburg, Germany), the resulting antibody complexes were visualized with diaminobenzidine (Sigma).

Using light microscopy the proportion of positively labeled cells and the staining intensity was quantified. A five step grading score was used for the proportion of stained cells (score 0: 0 cells stained; score 1: 0 – 25 cells stained; score 2: 26 – 50 cells stained, score 3: 51 – 75 cells stained; score 4: >75 cells stained) and a four step grading for the staining intensity (score 0: no positive staining; score 1: faint positive staining; score 2: moderate staining; score 3: strong staining intensity). 10 randomly selected areas (each 0.41 mm^2) on two different slides of each cell line were evaluated.

This score was calculated by multiplication of the amount of positively labeled cells and the staining intensity.

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